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Recovery of Transgenic Plants by *Agrobacterium*-mediated Genetic Transformation in *Dianthus caryophyllus* L. (carnation)

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ABSRTACT

Dianthus caryophyllus L. cv. `Tempo' is an important cut flower in the world. We used leaves as target tissue for genetic transformation. The efficiency of two regeneration systems with insect resistance (cry 1AB) gene was compared, through Agrobacterium tumefaciens mediated transformation. 100 mg/l kanamycin was selected for transformation. The transformation frequency was greatly affected by pre-conditioning and co-cultivation duration. The highest transformation frequency17.67% was obtained using callus regeneration system. The presence and expression of the foreign gene was confirmed by PCR and insect toxicity bioassay. The selected transformants showed normal phenotypes under in vitro and in vivo conditions.

Key words: *Agrobacterium tumefaciens*, carnation, genetic transformation.

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is one of the most important commercial cut flowers of the world. In U.S.A., it ranks next to rose in popularity. Carnation is cultivated widely on a large scale in Italy, Spain, Columbia, Kenya, Sri Lanka, Canary Islands, France, Holland, Germany and India. Being an important commercial crop, application of plant tissue culture and plant genetic engineering in carnation cultivars is of special value to obtain improved traits like disease and insect resistance. Carnation is severely affected by *Helicoverpa armigera*, which causes a considerable economic loss (40.9 to 91%) by infesting flower buds [1]. Modern biotechnological tools could be of much significance to alleviate the negative effects of chemicals and biopesticides, which are generally used to control insect pests. The use of transgenic crops, expressing foreign genes could be an important aspect of integrated pest management [2].

In carnation, the classical flower breeding is limited and being a vegetatively propagated crop, it further limits the available gene pool. This makes them ideal target for gene transfer technologies that have the potential to hasten the production of new genotypes and broaden the available gene pool [3]. Genetic transformation provides an alternative means for elucidating gene function and for making targeted single trait improvement in clonally propagated plants. Two requirements

for successful transformation are the ability to introduce desirable genes into the genome and the capacity to regenerate plants from the transformed cells [4].

Regeneration in carnation from callus explants has been reported to be difficult and depends upon the genotype, explant and plant growth regulators [5]. To apply genetic transformation technology to *Dianthus* species, it is essential to develop an efficient plant regeneration system. In this genus only carnation has been used as target for transformation since the first successful reports of Lu et al. [6] using *Agrobacterium*-mediated methods with stem explants, and by inducing transgenic shoots directly from the explants. Subsequent studies also used direct plant regeneration systems from various explants [7-11]. There have been no reports on the use of callus or cell suspension cultures for transformation in the genus *Dianthus*, however, Shiba and Mii [12] reported genetic transformation through cell suspension culture in *Dianthus acicularis*. Several reports are devoted to the development of regeneration systems for *Dianthus* species [10, 13, 14]. This paper describes comparison of two regeneration systems to find out the more applicable for *Agrobacterium*–mediated genetic transformation with insect resistance gene in *Dianthus caryophyllus* cv. `Tempo'.

MATERIALS AND METHODS

***Plant material*:**
Stem cuttings of carnation *(Dianthus caryophyllus* L.) cv. `Tempo' were obtained from the Department of Floriculture and Landscaping, University of Horticulture and Forestry, Solan (H.P.), India. Leaf segments were isolated from cuttings and used as explants. The leaf segments (0.5 cm^2) were treated with 0.1% carbendazim (Indofil, Bombay, India) solution for 10-15 min followed by washing under running tap water for 30 min. The explants were surface sterilized with 0.5% sodium hypochlorite solution for 15 min followed by 3-4 washings with sterilized distilled water.

Two regeneration systems were compared to find out the better one for genetic transformation studies.

Regeneration through callus culture:
The explants were inoculated onto MS [15] medium supplemented with 2,4-D (2 mg/l), BA (1 mg/l) and sucrose (30 g/l). The pH of the medium was adjusted to 5.8 followed by the addition of 0.8% agar. The medium was autoclaved at 121^oC and 1.1 Kg/cm^2 for 15 min. This medium was named as callus induction medium (CIM). The callus obtained after four weeks of culture was transferred to shoot regeneration medium (SRM) containing TDZ (2 mg/l) and IAA (1 mg/l) for shoot regeneration.

Direct adventitious shoot *regeneration*:
Direct shoot regeneration was achieved by inoculating explants onto MS medium (pH 5.8) supplemented with TDZ (2 mg/l), NAA (1 mg/l) and 30 g/l sucrose and solidified with 0.8% agar. This medium was designated as DSRM. The cultures were kept in dark for two weeks and transferred subsequently to 16:8 h photoperiod for direct shoot regeneration.

Shoots obtained from both regeneration systems were elongated and multiplied on the medium containing BA (1 mg/l), sucrose (30 g/l) and solidified with 1% agar (SEM). The shoots (3-4 cm) developed after four weeks were transferred onto rooting medium (1/2 MS + 2 mg/l IBA and 0.2% activated charcoal) in 25 x 150 mm culture tubes (Borosil). The rooting medium was referred as RM.

All the cultures were maintained at 25±2^{o}C under 16:8 h photoperiod (unless specified for dark incubation) at photon flux density of 50-60 µmol/m^{2}/s. The shoots with well developed roots obtained after four weeks of culture were transferred to earthen pots (10 cm) filled with sand:soil:farm-yard-manure (FYM) mixed in the ratio 1:1:1 for hardening in the glass house. The plants were acclimatized by gradually reducing the humidity.

Agrobacterium strain and plasmid:
Disarmed *Agrobacterium tumefaciens* strain EHA 105 harbouring binary vector pBin BtI was used for transformation [16]. The plasmid contains insect resistance (*cry*1Ab) and kanamycin resistance (*npt*-II*)* genes driven by CaMV and NOS promoters, respectively.

Kanamycin sensitivity test:
Pre-weighed explants were inoculated on callus induction and direct shoot regeneration media. Before inoculation, the media were supplemented with 10, 20, 30, 40, 50, 100 or 200 mg/l kanamycin (sterilized by Milipore membrane filter, 0.22 µm pore size). The observations on fresh weight increase were made at weekly interval till five weeks of culture.

Pre-conditioning of explants:
To observe the effect of pre-conditioning duration, the explants were inoculated on CIM and DSRM and incubated for 24, 48, 72 and 96 h.

Co-cultivation of explants:
The fresh culture of *Agrobacterium tumefaciens* was prepared by overnight growth of a single colony at 28^{o}C in 10 ml liquid yeast mannitol bacterium (YMB) medium [17] containing kanamycin (50 mg/l). The bacterial culture was centrifuged at 5000 rpm for 10 min. The pellet was resuspended in 1- 2 ml liquid MS basal medium to obtain a density of 10^{8} cells per ml (OD_{540} = 0.520). For infection, pre-conditioned explants were dipped in freshly prepared bacterial suspension for 10 min. After blotting away the excessive bacterial culture, the explants were placed again on the same media used for pre-conditioning and incubated for 24, 48, 72 and 96 h in dark for co-cultivation.

Selection and regeneration from callus:
The co-cultivated explants were washed in MS basal liquid medium, blotted dry on sterile filter paper and transferred to selective CIM containing100 mg/l kanamycin (HiMedia, Bombay, India) and 500 mg/l cefotaxime (Ranbaxy, India). Initially the cultures were subcultured for 2-3 times at weekly interval to avoid excessive bacterial growth. After four weeks, the calli were subcultured to fresh medium for callus proliferation and transferred subsequently on selective SRM having 100 mg/l kanamycin and 500 mg/l cefotaxime. Percent shoot regeneration and average number of shoots per callus were quantified after four weeks of inoculation.

Selection and regeneration of direct adventitious shoots:
The co-cultivated explants were washed, blotted dry and cultured on selective DSRM containing kanamycin (100 mg/l) and cefotaxime (500 mg/l). The cultures were incubated in dark for two weeks and transferred to light subsequently for direct adventitious shoot regeneration. The percent shoot regeneration and average number of shoots per explant were quantified after four weeks of culture. The putative transformed shoots obtained from both the regeneration systems were transferred to selective medium supplemented with BA (1 mg/l) and kanamycin (100 mg/l) for shoot elongation. The escapes (non-transformants) were removed by subculturing the green shoots on the respective selective media at an interval of four weeks (two cycles, 4 week/cycle). The kanamycin resistant shoots were multiplied on MS medium supplemented with BA (1 mg/l)

and l kanamycin (100 mg/). The shoots were rooted on selective RM containing l kanamycin (50 mg/) and hardened, as described earlier. After six weeks, percent survival of plants was recorded.

All the cultures were maintained at 25±2^{o}C with 16:8 h photoperiod (50-60µmol/m^{2}/s), unless specified for dark incubation. The experiment was repeated thrice .

Polymerase chain reaction analysis:

The PCR amplification was conducted on genomic DNA isolated from the leaves of kanamycin resistant shoots following the method of Offringa and Lee [18]. The DNA was amplified through thermal cycler (Perkin-Elmer, USA). A forward primer 5'-CCCGCTCCTCTCCGTCTACGTCC-3' and a reverse primer 5'-GGGCCCCTTCACCACCGATGTTCC-3' were used to identify the presence of *cry*1Ab gene. Each PCR reaction mixture (20 µl) consisted of 10.2 µl Milli Q water, 0.4 µl of 2U *Taq* DNA polymerase, 2 µl of 10X Taq DNA polymerase buffer, 1.8 µl of 25 mM $MgCl_2$, 1.6 µl of 200 mM dNTPs, 1 µl of 5 pM each primer and 2 µl of 50 ng genomic DNA. The conditions for PCR reactions were; one cycle at 94^{o}C for 4 min as pre-heating, 32 cycles at 94^{o}C for 60 sec (denaturation) 55^{o}C for 60sec (annealing), 72^{o}C for 120 sec (extension) and one cycle at 72^{o}C for 5 min (final extension). Plasmid DNA used in transformation served as a positive contol while DNA from non-transformed plants was used as a negative control. The amplified products were separated on 1.4% agarose gel and stained with ethidium bromide to visualize with using a UV transilluminator and photographed using gel documentation system (Alpha Imager, USA). *Taq* DNA polymerase, 10X buffer, dNTPs and 25 mM $MgCl_2$ were obtained from Bangalore Genei, Bangalore, India.

Bioassay for insect toxicity:

Eggs of *Helicoverpa armigera* were hatched and second instar larvae were reared on an artificial diet consisting of chickpea soaked in water for 48 h. The tests were conducted using excised leaf tissue from control and transformed plants.. The visual observations on the feeding and mortality rate of larvae were recorded daily till one week. The bioassay experiment was repeated three times.

Statistical Analysis:

The experiments were conducted in a completely randomized design (CRD). The data recorded on different parameters were subjected to analysis of variance (ANOVA) using CRD [19]. Data transformation was carried out as needed to satisfy ANOVA requirements. Once significant treatment effects were found, Fischer's least significant difference (LSD, P≥0.05) was used to compare the means.

RESULTS AND DISCUSSION

Kanamycin sensitivity test:

The test revealed that the kanamycin at a concentration of 100 mg/l was optimum for selection of explants inducing callus or regenerating shoots directly. In kanamycin free medium (control), the explants were healthy and produced callus with maximum increase in fresh weight after five weeks. Concentration above the optimal level turned the material completely necrotic with negligible increase in fresh weight. These results conformed the earlier findings [6, 9, 20] but contrary to those [21] who made selection at kanamycin (250 mg/l).

Effect of pre-conditioning and co-cultivation duration on transformation frequency:

Pre-conditioning and co-cultivation had a marked effect on the production of kanamycin resistant material**.** Co-cultivation of the explants for 96 h preceded by 96 h pre-conditioning resulted in

highest percentage of kanamycin resistant leaf explants producing callus (17.67%) whereas 96 h pre- conditioning followed by 72 h co-cultivation resulted in highest percentage of explants regenerating shoots buds directly (1.66%). The comparison of two regenerating systems for producing higher percentage of kanamycin-resistant material clearly showed the superiority of callus phase regenerating system over the other as it gave maximum transformation rate in the presence of kanamycin (**Tables -1 and 2**). The percentage of explants growing well on the selective medium was calculated as transformation frequency.

Table 1. Effect of pre-conditioning and co-cultivation duration on leaf explants inducing callus and putative transformation rate after one month of culture on selective medium

Pre-conditioning duration (h)	Co-cultivation duration (h)	Number of explant treated	Number of explants inducing callus on selective medium	Putative transformation rate (%)
24	24	15	0.00(1.00)	0.00(1.00)
24	48	15	0.00(1.00)	0.00(1.00)
24	72	15	0.00(1.00)	0.00(1.00)
24	96	15	0.00(1.00)	0.00(1.00)
48	24	15	0.00(1.00)	0.00(1.00)
48	48	15	0.00(1.00)	0.00(1.00)
48	72	15	0.00(1.00)	0.00(1.00)
48	96	15	0.00(1.00)	0.00(1.00)
72	24	15	0.00(1.00)	0.00(1.00)
72	48	15	0.85(1.21)	5.66(2.58)
72	72	15	1.29(1.52)	8.66(3.10)
72	96	15	1.60(1.62)	10.67(3.41)
96	24	15	0.00(1.00)	0.00(1.00)
96	48	15	1.24(1.50)	8.33(3.05)
96	72	15	2.44(1.82)	16.33(4.16)
96	96	15	2.65(1.91)	17.67(4.32)
$CD_{0.05}$			(0.05)	(0.02)

Figures within parentheses are arc sine transformed values

Table 2. Effect of pre-conditioning and co-cultivation duration on adventitious shoot bud induction and putative transformation rate after one month on selective medium

Pre-treatment duration (h)	Co-cultivation duration (h)	Number of explants treated	Number of explants inducing shoots buds	Number of shoot buds per explant	Putative Trans-Formation (%)
24	24	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
24	48	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
24	72	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
24	96	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
48	24	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
48	48	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
48	72	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
48	96	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
72	24	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
72	48	30	0.37(1.25)	0.96(1.40)	1.23(1.49)
72	72	30	0.32(1.15)	1.03(1.42)	1.06(1.43)
72	96	30	0.43(1.12)	1.23(1.49)	1.43(1.56)
96	24	30	0.00(1.00)	0.00(1.00)	0.00(1.00)
96	48	30	0.49(1.19)	1.36(1.53)	1.63(1.62)
96	72	30	0.50(1.21)	1.33(1.52)	1.66(1.63)
96	96	30	0.49(1.19)	1.43(1.33)	1.63(1.62)
$CD_{0.05}$			(0.02)	(0.02)	(0.01)

Figures within parentheses are square root transformed values

Co-cultivation period of more than three days have been successfully used in carnation cultivars [6, 22, 23]. However, 24 h co-cultivation duration preceded by 72 and 96 h pre-conditioning did not produce any response indicating that transformation process was not completed within this duration. Similarly, 24, 48, 72 and 96 h co-cultivation preceded by 24 and 48 h pre-conditioning also yielded no response (**Tables -1 and 2**).

In the present study, the explants were wounded before co-cultivation for pronounced wound response. The explants were dipped in *Agrobacterium* suspension for 10 min and kept for co-cultivation in dark. The gene products encoded by vir region of Ti plasmid are induced in the bacterium during co-cultivation [24] when it comes in contact with the phenolic exudation from the wounded edge of the explant, resulting in the transfer of T-DNA into the plant genome [25]. The co-cultivated explants were transferred onto respective selective media for callus induction and direct shoot regeneration.

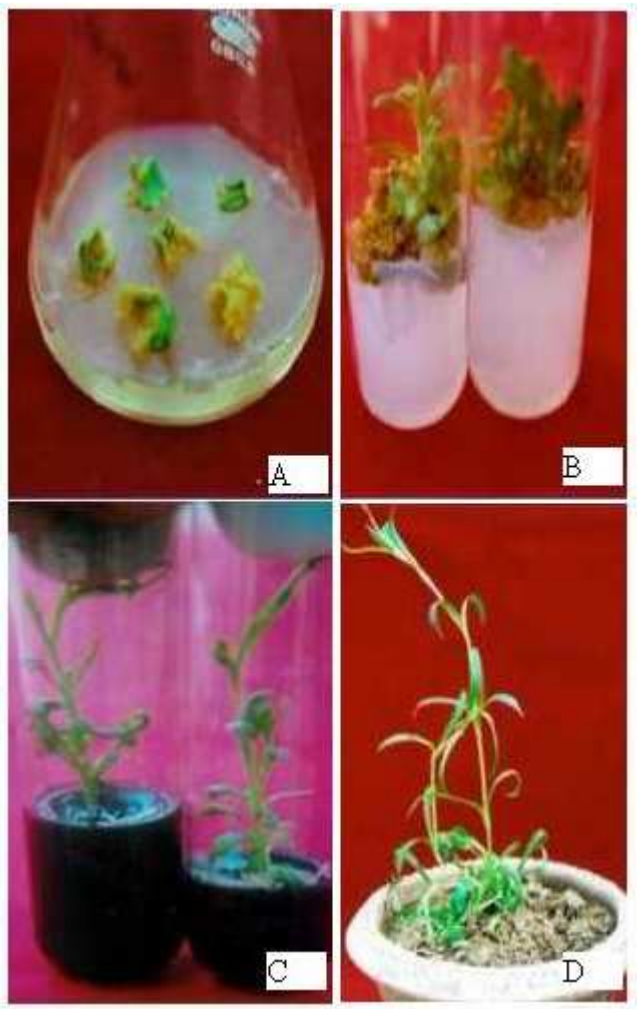

Fig – 1. Regeneration of transgenic plants from leaf callus.
A. *Callus on selective CIM after two weeks.*
B*. Shoot regeneration from leaf derived calli on selective shoot regeneration medium.*
C*. Rooted plantlet on selective rooting medium.*
D*. Hardened transgenic plants after six weeks.*

Selection and regeneration from callus:
Maximum percentage of kanamycin resistant material was obtained in the explants pre-conditioned for 96 h followed by 96 h co-cultivation, therefore further observations were

restricted to the explants of this treatment (**Table - 1**). Within two weeks on the selective medium, an average of 17.67% explants showed callus induction and within four weeks the whole surface of the explants was covered with (**Fig. -1A**). The control and some of the co-cultivated explants become completely necrotic within two weeks of culture on selective medium.

The growing callus on putatively transformed explants was sub-cultured and 6.13% of growing callus differentiated and showed shoot bud induction with an average of 2.90 shoots/callus (**Fig. – 1B**). The shoots thus obtained were green coloured and none of them were necrotic, indicating the presence of *cry*1AB gene and indicating that regeneration has occurred only from transformed cells of callus, while non-transgenic callus turned dark and did not show regeneration. Lu et al. [6] transformed carnation and found that out of 144 co-cultivated stem segments, 12 grew well on selective medium and showed GUS activity.

On selective rooting medium 71.33% shoots induced roots (**Fig. – 1C**) and the plantlets thus obtained showed 80% survival upon transfer to pots (**Fig. – 1D**). All transformed plants showed normal phenotype *in vitro* and under glasshouse conditions.

Fig – 2. Regeneration of transgenic plants by direct adventitious shoots from leaf explant.
A. *Direct shoot regeneration from leaf explants on selective DSRM after four weeks.*
B*. Shoot multiplication on selective shoot multiplication medium.*
C*. Rooted plantlet on selective rooting medium.*
D*. Hardened transgenic plant after six weeks.*

Selection and regeneration of direct adventitious shoot buds:

The observations were made for the explants co-cultivated for 72 h preceded by 96 h pre-conditioning (**Table - 2**). Four weeks (two weeks in the dark followed by two weeks to light) following transfer to the selective medium, an average of 1.66% co-cultivated explants showed

shoot bud induction with 1.36 shoot bud per explant (**Fig. – 2A**). The explants surviving selection pressure were transferred to light (16:8 h photoperiod). Shoots were elongated on selective elongation medium (SEM) and subjected to two selection cycles to remove escapes. After four weeks the shoots were separated and cultured individually on the fresh shoot multiplication medium (**Fig. -2B**).

In this study, kanamycin served as an efficient selective agent where the escapes or non-transformed tissue showed loss of chlorophyll and can easily be removed. All the green shoots growing on the selective medium were found transformed when assayed by PCR.

Root induction started within two weeks and complete development of roots was observed within four weeks (**Fig. – 2C**) resulting in 70.33% rooting. Upon transfer to pots the survival rate of plantlets was 82.17% (**Fig. – 2D**).

In comparison, direct shoot regeneration system has been used earlier for genetic transformation in carnation [8-11] as it does not involve long de-differentiation phase and the problem of chimeral shoots due to somaclonal variations is reduced. In the present study, the callus-mediated regeneration system seems to be promising and may help in carnation improvement programs as higher transformation frequency was observed in this regeneration system than the direct shoot regeneration system.

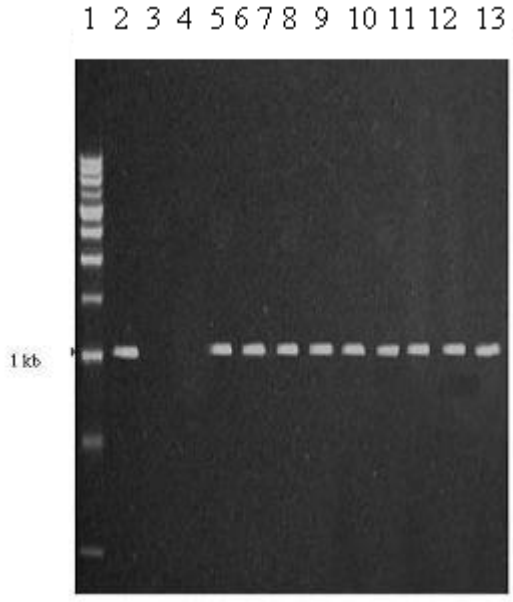

Fig – 3. PCR analysis of genomic DNA isolated from leaves of non-transformed and transformed shoots.
Lane 1 = A 500 bp DNA ladder.
Lane 2 = Plasmid (positive control).
Lanes 3 and 4 = DNA samples from non-transformed controls.
Lanes 5-8 = DNA samples from callus-derived transformants (T_1-T_4)
Lanes 9-13 = DNA samples from direct shoot bud induction transformants (T_5-T_9).

Polymerase chain reaction analysis:

The PCR analysis revealed 1kb *cry1Ab* gene amplification from genomic DNA isolated from all kanamycin resistant shoots DNA samples showed amplified gene bands with *cry1Ab* gene specific primers, thereby indicating the presence and integration of *cry1Ab* gene in these nine shoots (T_1 –T_9). No amplification products were observed for non transformed control shoots (**Fig - 3**). The transformation frequency was still low probably the recalcitrant nature of the

carnation cells affects transformation. The recalcitrant nature of cells affecting transformation was reported in *Hypericum perforatum* [26]. No reports were available on the use of callus or cell suspension cultures for transformation because of the difficulty in regenerating plants from highly de-differentiated cultures in carnation. The transformation frequency of 2.5% was reported in *Agrobacterium* mediated transformation of petal explants of carnation cv. `White Sim' [23]. The expression of the gene was further confirmed by an insect bioassay.

Bioassay for insect toxicity:

The *cry1Ab* gene expression in PCR positive plants was confirmed by insect bioassay. All the transformed plants showed strong expression of insect resistance gene, inferred from lesser feeding of the leaves by the larvae as compared to control. 100% mortality was observed in 7 days of initiation of bioassay experiment (**Table - 3**).The degree of insect protection conferred by the expression of the *cry1AB* protein in transformed carnation was significant as shown by insect bioassay.

Table 3. Percent mortality in second instar larvae of *Helicoverpa armigera* in transgenic plants

Days	Mortality (%)*			
	Callus regeneration system		Direct shoot regeneration system	
	Control	Transgenic	Control	Transgenic
1	0	0	0	0
2	0	0	0	0
3	0	26.7	0	13.3
4	0	46.7	0	33.3
5	0	73.3	0	66.7
6	0	100	0	86.7
7	0	-	0	100

**Mean of three experiments. Five larvae per experiment in each system*

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